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Microparticles and Their Impact on Intestinal Immunity

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Inflammasome · Microparticles · Interleukin-18

Abstract
Microparticles are small (<1 μm), nonbiological particles that are used in many areas of daily life. As food additive they are used as anticaking agents or food colorants. The most common food-derived ingested compounds are aluminium silicate and titanium dioxide (TiO_2), the latter being a white pigment used in toothpaste or sugar toppings. The increasing abundance of microparticles in the Western diet raises the question of the potential risks associated with gastrointestinal diseases such as Crohn's disease (CD). Accumulation of particles has been shown in cells of Peyer's patches, but it is not clear whether this also has pathological effects. NLRP3 is a member of the intracellular pattern recognition receptor family and it is part of the inflammasome, a multiprotein complex containing caspase-1 which activates the proinflammatory cytokines interleukin (IL)-1β and IL-18. With regard to recent findings identifying small particles such as asbestos and monosodium urate as NLRP3 activators, TiO_2 may be another potential target for inflammasome studies. We found that macrophage-like cells readily take up TiO_2 after 6 h. Incubation of cells with TiO_2 resulted in the assembly of NLRP3 with caspase-1. This inflammasome assembly correlated with secretion of IL-1β. In intestinal epithelial cells, TiO_2 also was found to be ingested. The counting of particles localized intracellularly revealed a dose-dependent increase of TiO_2-positive cells. This points to the fact that in humans with a leaky intestinal barrier (such as IBD patients), TiO_2 microparticles may be taken up by macrophages and intestinal epithelial cells, may activate the inflammasome and induce IL-1β and IL-18 secretion. This may aggravate inflammation in susceptible individuals.

Introduction

Intestinal epithelial cells (IECs) form a single layer on the surface of the intestinal mucosa and the primary barrier against the content of the gut lumen. They protect the underlying tissue and the entire organism from potentially harmful microorganisms, particles or larger molecules. IECs secrete antimicrobial substances such as defensins [1, 2] and communicate with the immune cells of the intestinal mucosa via a high number of soluble mediators such as chemokines and cytokines. In patients with inflammatory bowel disease (IBD), the epithelial and mucosal barrier function is disturbed, finally leading to a chronic relapsing inflammation of the intestinal mucosa.

Both environmental triggers and genetic susceptibility have been shown to contribute to the pathophysiology of IBD. More than 140 susceptibility loci for IBD have been identified mainly by genome-wide association stud-

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ies (GWAS) [3], among them a number of genes related to innate immune functions including single-nucleotide polymorphisms (SNPs) in so-called pattern-recognition receptors (PRRs) which recognize microbial patterns (i.e. pathogen-associated molecular patterns, PAMPs) but also other molecular signatures that may be a risk for health (so-called danger-associated molecular patterns, DAMPs).

In healthy individuals, activation of PRRs and the subsequent proinflammatory signalling is tightly regulated. Among other members of the PRR family, NLRP3 [nucleotide-binding domain-containing and leucine-rich repeat-containing (NLR) family and pyrin domain-containing 3 (PYD)], has been shown to play a role in IBD etiology. Alternative names for NLRP3 are NALP3, CIAS (cold-induced autoinflammatory syndrome 1), PYPAF (PYD-containing Apaf1-like) and cryopyrin. In biopsies of Crohn's disease (CD) patients, NLRP3 expression was found to be increased [4]. Besides microbial patterns, NLRP3 also recognizes 'danger signals' or DAMPS such as adenosine triphosphate (ATP) [5], asbestos [6] and uric acid crystals [7]. Upon activation signals, NLRP3 gets associated with a large multiprotein complex, the NLRP3 inflammasome, which activates caspase-1. Activated caspase-1 then finally cleaves pro-interleukin (IL)-1β and pro-IL-18 (fig. 1).

Expression of all components of the NLRP3 inflammasome has been observed in professional immune cells such as macrophages or dendritic cells, but a high level has also been found in IECs.

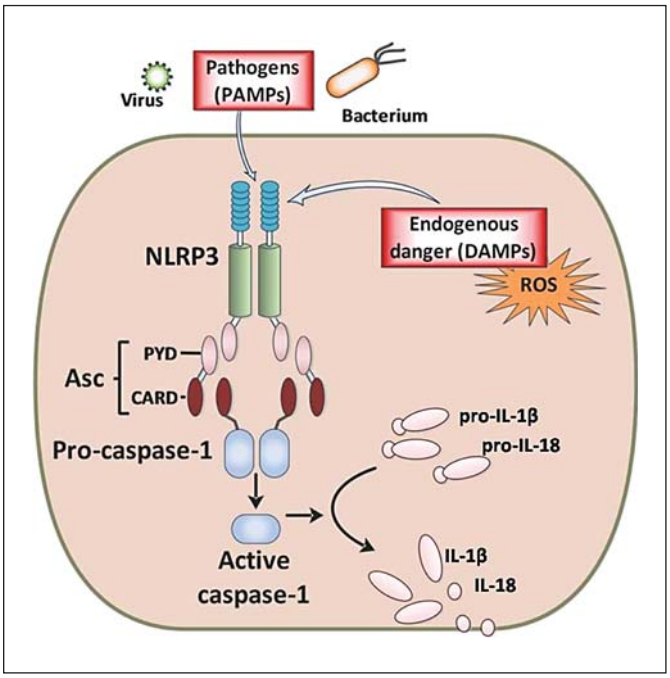

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Fig. 1. NLRP3 inflammasome activation. PAMPs and DAMPs such as ROS have been shown to activate the NLRP3 inflammasome. Upon activation, NLRP3 assembles to a multiprotein complex together with the adaptor protein, apoptosis-like speck protein (Asc) and caspase-1. Caspase-1 undergoes autocatalytic activation, which enables the cleavage of its substrates pro-IL-1β and pro-IL-18. The cleaved cytokines are then released by the cell to activate downstream targets.

NLRP3 and Susceptibility to IBD

Similar to nucleotide-binding oligomerization domain-containing protein 2 (NOD2), the first-identified CD susceptibility gene, NLRP3, contains a 'caspase-recruitment domain' (CARD) and, upon activation, assembles with caspase-1 to form the inflammasome [8]. The relevance of NLRP3 for CD pathogenesis is obvious from the finding that a gain-of-function SNP in NLRP3 and a correlating CARD8 loss-of-function SNP both result in a protective effect against CD [9, 10]. NLRP3 gain-of-function mutations were previously associated with other inflammatory diseases and syndromes such as rheumatoid arthritis [11], Muckle-Wells syndrome, familial cold autoinflammatory syndrome [12] and chronic infantile neurological cutaneous and articular syndrome [13]. All are characterized by elevated IL-1β levels, indicating an NLRP3/inflammasome activation. Further, NLRP3 was shown to be upregulated in colon tissue after the induction of acute or chronic colitis in mouse models. An elevated expression was also found in colon specimens of CD patients [4].

The inflammasome proteins belong to a family of NLRs, all sharing common domains that act as intracellular sensors for PAMPs and DAMPs. Similar to Toll-like receptors, the NLR family is evolutionarily ancient and shares structural homologies with plant resistance (R-) proteins. Both protein families have leucine-rich repeat (LRR) regions for the recognition of bacterial motifs, an oligomerization domain and an effector domain [14] (fig. 2).

Upon activation, NLRP3 assembles into a multiprotein complex, recruiting other NLRP3 proteins, CARD8, ASC (apoptosis-like speck protein) and caspase-1 (fig. 1). The recruitment of caspase-1 triggers its autocatalytic cleavage and activation. Subsequently, caspase-1 cleaves pro-IL-1β or pro-IL-18; this is usually followed by the release of biologically active forms of these cytokines (table 1).

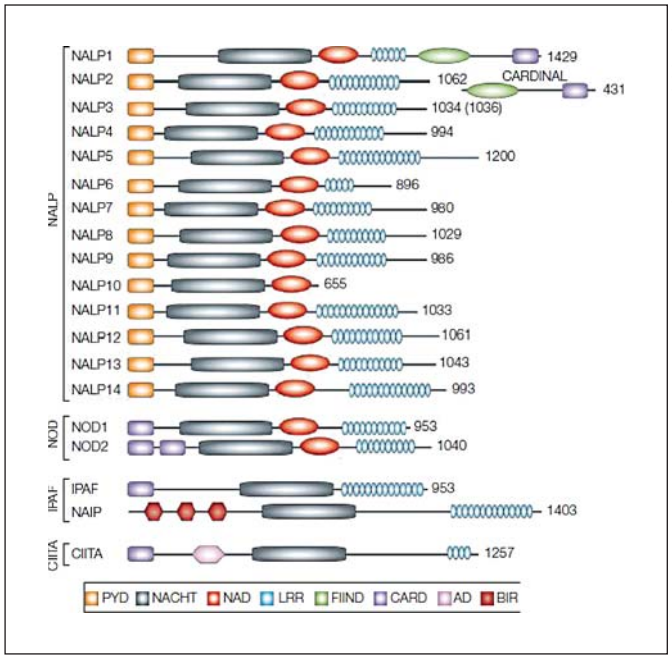

Fig. 2. Domains of the NLR family. In the NLRP subfamily, the PYD enables interaction with other proteins, whereas NOD1, NOD2, ice protease-activating factor (IPAF) and MHC class II transactivator (CIITA) contain CARD domains for interaction. Furthermore, LRRs are found at the C-terminus of all NLR family members. AD = Acid transactivation domain; BIR = baculovirus inhibitor of apoptosis repeat; FIIND = domain with function to find; NACHT = domain present in NAIP, CIITA, HET-E, and TP-1; NAD = NBD-associated domain; NAIP = NLR family, apoptosis inhibitory protein; NALP = NLRP [66].

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Table 1. Activators of the NLRP3 inflammasome (table adapted from [15])

		Reference
Microorganisms		
Sendai virus	09/2006	[16]
Influenza virus	09/2006, 04/2009	[16, 17]
Adenovirus	03/2008	[18]
Staphylococcus aureus	03/2006, 09/2009	[5, 19]
Listeria monocytogenes	03/2006, 05/2008	[5, 20]
Saccharomyces cervisiae	06/2009	[21]
Candida albicans	08/2009	[22]
Aspergillus fumigatus	04/2010	[23]
Bacterial RNA or synthetic purine bases	03/2006	[24]
Bacterial pore-forming toxins (aerolysin, maitotoxin and nigericin)	03/2006, 09/2006, 11/2006	[5, 25, 26]
Malaria toxin	08/2009	[27]
Danger signals		
Extracellular ATP	03/2006	[5]
Hyalorunan	03/2009	[28]
Glucose	12/2009	[29]
Monosodium urate	03/2006	[7]
Amyloid-beta	08/2008	[30]
Silica	08/2008, 07/2008	[31, 32]
Asbestos	07/2008, 05/2008	[6, 32]
Alum	08/2008	[31]
Cholesterol crystals	04/2010	[33]

Food-Derived Environmental Factors in the Pathogenesis of IBD?

IBD cannot only be attributed to genetic factors, as monozygotic twins have a concordance rate of only 20–50% of developing IBD [34–37]. The increased incidence of IBD in the last 100 years, the higher incidence of IBD in countries with a Western lifestyle and the observed rise of IBD in countries recently undergoing 'westernization' in Asia have led to several hypotheses explaining this phenomenon, summarized by Hanauer [38]. Hygiene, diet, social status and smoking are factors potentially influencing the risk for developing IBD [39]. Some childhood factors such as breastfeeding and childhood infections are likely to have protective functions, and it has been suggested that increased hygienic standards increase the risk of IBD. Another factor associated with a typical Western diet is microparticles.

Microparticles are used as food additives in order to influence the color, the consistency or the appearance of a product. The most commonly used additives are titanium dioxide (TiO_2, E171) and aluminium silicate (AlSi, E559). Both compounds are sub-micron-sized (0.1–1 μm diameter) oxides of titanium, silicon or aluminium. Since they are inorganic, they are highly stable and resistant to degradation. According to current European legislation, there is no maximum TiO_2 level specified. It is recommended that TiO_2 should be used in amounts according to good manufacturing practice [40]. The international agency for research on cancer published an overview on studies addressing the hazardous potential of TiO_2 in 2006. In animal studies, inhalation was followed by the development of tumors. In vitro studies with human alveolar macrophages revealed inhibited phagocytosis and DNA damage. Subsequently, TiO_2 was rated as possibly carcinogenic for humans (Group 2b) [41]. Classification by the International Agency for the

Research on Cancer, IARC, as Group 2B. This means that the agent is possible carcinogenic in humans but there is limited evidence from either animal experiments or human studies. In the case of TiO2 there is evidence from animal studies but limited evidence from human studies.

Different Microparticles Used in the Food Industry

TiO_2 is a white, crystalline powder which is widely used as a food supplement in refined food. The pigment is used in toothpaste, confectionery, white sauces and dressings and nondairy creamers [42]. In the pharmaceutical industry, it is used as a pill coating. It is inert to most influences (fig. 3).

Iron Oxide

Iron oxide (Fe_2O_3) is a nonsoluble, brown and red pigment. It is used as a food colorant as well as in cosmetics. The product name is 'Red luster' which is also used for coating pharmaceutical formulations.

Titanium Dioxide/Iron Oxide Mixture

TiO_2/Fe_2O_3 mixture is a gold mica powder. The powder is used as a food colorant and as a coating for pharmaceutical formulations.

AlSi

AlSi, also known as kaolin, is a natural white, fine-grained material. The field of application includes the paper-manufacturing industry and powder fabrication, but it is also used in the food industry as a carrier substance, bleaching product and parting agent, as it prevents caking (fig. 3).

Titanium Oxide/AlSi Mixture

TiO_2 with potassium (K+) and AlSi added is used as a whitener (fig. 3).

Sources of Dietary Microparticles

Microparticles are not declared in the contents of many products. The daily intake is difficult to evaluate and is highly dependent on the individual diet. According to a study by the European Food Safety Authorities (EFSA), the estimated daily intake of TiO_2 is 70–80 mg/day [43]. Sources may be tablets, milk whitener and toothpaste among others. Other authors estimate the daily ingested amount of both TiO_2 and AlSi to be 40 mg or 10^{12} particles/person/day [44, 45].

An overview of sources and estimated amounts of microparticles is given in table 2 adapted from [43, 44].

Effects of Titanium Dioxide on Intestinal Epithelial Cells

Microparticles may penetrate the intestinal barrier and accumulate in the mucosa. Early reports on dark, pigmented structures in human intestinal lymphoid aggregates date back to 1987 [46]. These structures were later found to contain high amounts of titanium [47]. TiO_2 particles have been shown to accumulate in M cells of Peyer's patches and are passed on to underlying macrophages [48]. Interestingly, the sites of TiO_2 uptake correspond to regions where the first signs of inflammation in CD manifest [49].

In vivo studies on the capacity of TiO_2 to penetrate the gastrointestinal tract revealed that TiO_2 can be found in systemic organs after an oral exposure of 10 days [50–52]. In humans, after a single ingestion of 22 mg and 45 mg of TiO_2, Ti levels in the blood rose after 0.5–2 h and reached a maximum after 8–12 h [52].

Clinical studies have also addressed the influence on TiO_2 on CD patients. A pilot study performed in 2002 observed significant decreases in the CD activity index [53] after 4 months on a low-TiO_2 diet. However, a larger multicenter double-blind trial was not able to confirm these results [54]. Under present living conditions, a reduction or exclusion of dietary TiO_2 may be very difficult. With the recent discovery of it being an activator of the NLRP3 inflammasome, the influence of TiO_2 on IEC in the healthy and in the chronically inflamed mucosa has gained new interest. Dendritic cells derived from NLRP3-deficient mice were shown to secrete less IL-1β upon exposure to TiO_2 [55]. In human keratinocytes and in murine macrophages, TiO_2 activated the NLRP3 inflammasome [56]. These data point towards a role of the inflammasome as a direct or indirect sensor of TiO_2.

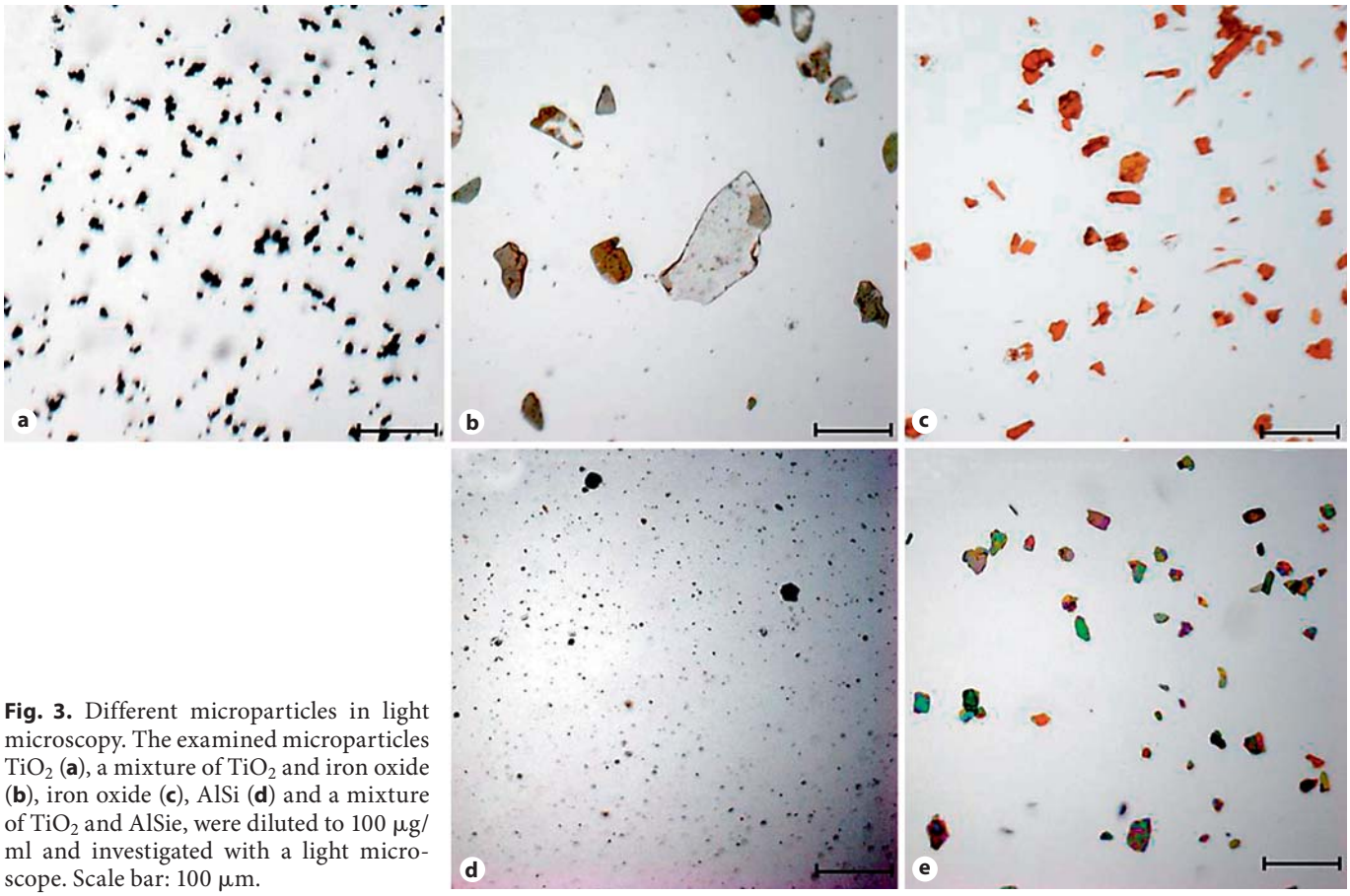

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Fig. 3. Different microparticles in light microscopy. The examined microparticles TiO_2 (**a**), a mixture of TiO_2 and iron oxide (**b**), iron oxide (**c**), AlSi (**d**) and a mixture of TiO_2 and AlSie, were diluted to 100 μg/ml and investigated with a light microscope. Scale bar: 100 μm.

Pathways of NLRP3 Activation

It is still not clear how the NLRP3 inflammasome is activated in detail. Some authors suggest an initial priming event via the NF-κB pathway [57]. The so-called DAMPs comprise a group of inflammasome activators, and several pathways and mechanisms have been shown to be involved in a multistep activation. Recently, Chen and Nunez [58] summarized the most important facts on DAMP-associated inflammasome activation. Three separate pathways have been suggested. The first activation pathway requires high levels of extracellular ATP. ATP may be released by apoptotic/necrotic cells and trigger the opening of the purinergic receptor P2X, ligand-gated ion channel 7 (P2RX7). This channel triggers the formation of pores in the cell membrane that allow an efflux of K+ ions. Inflammasome activation can be prevented by high concentrations of extracellular K+ [32, 59].

Table 2. Sources of microparticles in food products and estimated daily intake

Source	mg/person/day
TiO_2	
Food supplement tablet	37.5
Confectionary	24.4
Medicinal product tablet	15.0
Coffee whitener	0.52
Hard coated candies	0.32
Chewing gum	0.28
Marshmallows	0.27
Low-fat or fat-free dressings	0.22
AlSi	
Salt	1.30
Drinking chocolate powder	1.26
Chewing gum	0.92
Sugar, icing	0.30

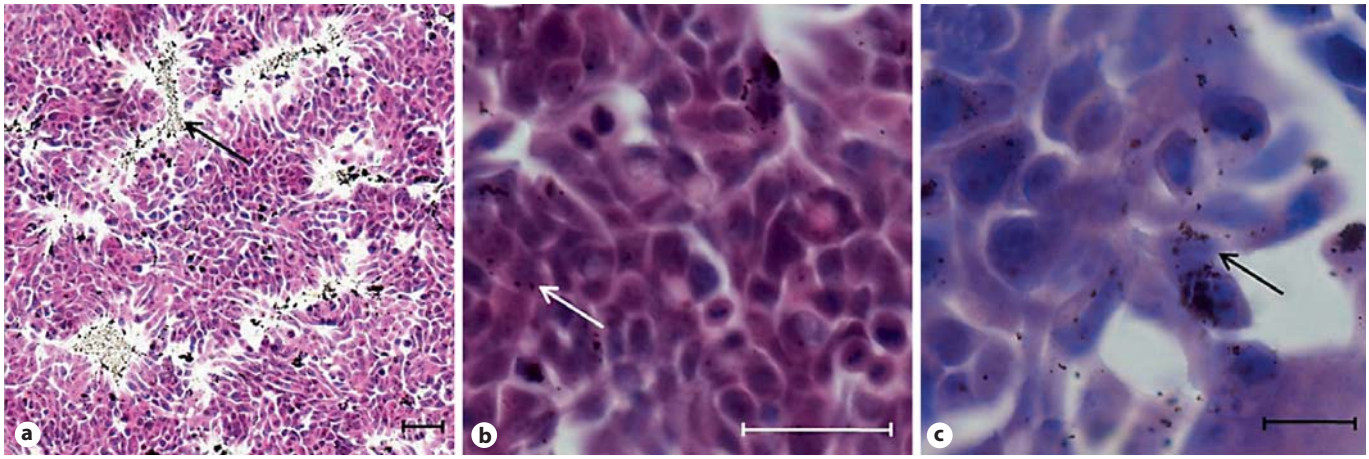

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Fig. 4. Eosin-stained cells stimulated with TiO_2. TiO_2 particles could be found intracellularly. The most frequent uptake of particles could be detected in cells at the margin of the monolayer. Scale bars: 50 μm (**a**, **b**) and 20 μm (**c**).

The second pathway may be relevant for uric acid crystals (similar to cholesterol crystals), which induce inflammasome activation by a different mechanism [7, 33]. These crystals are taken in lysosomes by cells such as macrophages. As they cannot be degraded, lysosomal damage occurs followed by release of the lysosomal protease cathepsin B which, in turn, triggers NLRP3 activation. The lysosomal rupture may cause the production of reactive oxygen species (ROS) [60, 61]. In line with this, the activation of the inflammasome, and subsequently caspase-1, in response to asbestos crystals was partially prevented when ROS synthesis was inhibited [6]. Release of ROS is a danger signal for surrounding cells, and NLRP3 has been shown to be activated by necrotic cells. In the third concept of inflammasome activation, ROS is regarded to be the most important trigger. ROS may act both as extracellular and intracellular danger signals.

Microparticles and Inflammasome Activation

Under in vitro conditions we found microparticles consisting of TiO_2 intracellularly in IEC and macrophages already at low concentrations (fig. 4). Their intracellular accumulation did not dependent on endocytosis. TiO_2 aggregates were not restricted to a specific subcellular structure as observed by electron microscopy. The incubation of macrophages and IEC with low concentrations of TiO_2 resulted in assembly of the NLRP3 inflammasome, and correspondingly, the downstream effectors caspase-1, IL-1β and IL-18 were activated and secreted. High doses of TiO_2 caused the generation of ROS in the tested cell types, as previously reported for other particulate structures [6]. In contrast to other findings with silica crystals [31], the uptake of TiO_2 did not lead to lysosomal rupture.

Increased levels of functionally active IL-18 in IEC from CD patients are indicative of an increased inflammasome activation. This is in line with findings of other groups reporting the transcriptional upregulation of IL-18 and increased protein levels of mature IL-18 in the mucosa of CD patients [62, 63].

TiO_2 has thus far mainly been the subject of studies focusing on effects of inhalation exposure. However, microparticles are also ingested on a daily basis in the Western diet and their use as food additive is on the rise and not limited by food laws. The long-term impact of dietary microparticles such as TiO_2 and AlSi has not been studied, and the risk evaluation is inconclusive. Given that TiO_2 is also used in pharmaceutical pills, one can assume that local luminal concentrations of TiO_2 can reach substantial amounts. Depending on the properties of the pills, they disintegrate in different sections of the gastrointestinal tract and may affect the intestinal epithelium in a very distinct and concentrated manner.

Further work should include other dietary microparticles such as AlSi and Fe_2O_3 and evaluate their capacity to activate the NLRP3 inflammasome and proinflammatory signalling in macrophages and in IEC. Preliminary data show that, for instance, Fe_2O_3 may be too large to penetrate the cells. An effect of TiO_2 on epithelial cells that has been reported by others was the reduction of transepithelial resistance [64, 65].

Excluding microparticles from food could be a way to improve the well-being of IBD patients. Therefore, it would be worthwhile to initiate further studies with IBD patients to reevaluate the impact of dietary microparticles on the disease activity.

Conclusion

The immune function of IEC is a large field of research, and it has become evident that these cells play an important role as a first line of defense, but also function as signal inducers for underlying immune cells. The tight regulation of proinflammatory and anti-inflammatory signalling is an important feature of IEC, which is out of balance in the state of chronic intestinal inflammations. The emerging role of genetic variations leading to an increased susceptibility to develop IBD is overwhelming, but the actions of many susceptibility genes are not fully understood.

The discovery of NLRP3 as a platform for the recognition of various microorganisms and the more recent revelations on the nonbacterial activators of NLRP3 have gained much attention in recent years. The identification of TiO_2 microparticles as activators of the NLRP3 inflammasome shows that not only immune cells, but also IEC are responsive to nonbacterial stimuli. The current data suggest that TiO_2 triggers inflammation, ROS and, at high concentrations, also apoptosis. Since TiO_2 is consumed as a white pigment in a number of foods and pharmaceutical pills, this finding may evoke questions about food safety, especially in the context of IBD. Further investigation in animal models but also in healthy individuals and IBD patients will help to reevaluate the risk of microparticles in our food.

Disclosure Statement

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